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The effect of mitragynine on cAMP formation and mRNA expression of *mu*-opioid receptors mediated by chronic morphine treatment in SK–N–SH neuroblastoma cell

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ABSTRACT

Ethopharmacological relevance: Mitragynine is an indole alkaloid compound of *Mitragyna speciosa* (*M. speciosa*) Korth. (Rubiaceae). This plant is native to the southern regions of Thailand and northern regions of Malaysia and is frequently used to manage the withdrawal symptoms in both countries.

Aim of study: To investigate the effect of mitragynine after chronic morphine treatment on cyclic AMP (cAMP) level and mRNA expression of *mu*-opioid receptor (MOR) in human neuroblastoma SK–N–SH cell.

Method and Materials: Mitragynine was isolated from the *Mitragyna speciosa* plant using the acid–base extraction method. The cAMP level upon forskolin stimulation in the cells was determined using the Calbiochem® Direct Immunoassay Kit. The mRNA expression of the MOR was carried out using quantitative RT-PCR.

Result: Cotreatment and pretreatment of morphine and mitragynine significantly reduced the production of cAMP level at a lower concentration of mitragynine while the higher concentration of this compound could lead to the development of tolerance and dependence as shown by the increase of the cAMP level production in foskolin stimulation. In MOR mRNA expression study, cotreatment of morphine with mitragynine significantly reduced the down-regulation of MOR mRNA expression as compared to morphine treatment only.

Conclusion: These finding suggest that mitragynine could possibly avoid the tolerance and dependence on chronic morphine treatment by reducing the up-regulation of cAMP level as well as reducing the down-regulation of MOR at a lower concentration of mitragynine.

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1. Introduction

Opioid receptors belong to the G protein-coupled receptor superfamily. In the nervous system, activation of opioid receptors leads to neuronal inhibition and causes several receptor–effector mechanisms, which includes activation of receptor-operated potassium channels, inhibition of voltage-gated calcium channels and inhibition of adenylyl cyclase (Law et al., 2000; Koch and Hollt, 2008) Pharmacological studies using highly selective ligands have classified opioid receptor into three subtypes; μ, δ and κ based upon a characteristic pharmacology. Morphine and other opioid peptides principally target or bind selectively with high affinity to the *mu*-opioid receptor (Yu et al., 2003). It is known that, the development of tolerance and dependence usually formed from the chronic administration of opiates such as morphine and heroin (Waldhoer et al., 2004; Christie, 2008; Berger and Whistler, 2010).

Several *in vitro* and *in vivo* studies revealed that development of opioid tolerance in long-term exposure with morphine will lead to downregulation of receptors or counterregulatory processes such as adenylate cyclase superactivation (Varga et al., 2003; Zhao et al., 2006). It is also shown that cyclic adenosine monophosphate (cAMP) and its signaling pathways play a crucial role in the processing of painful stimuli. The mechanism involved in increasing and decreasing the cAMP level is generally associated with increased nociception and have an analgesic effect (Finn and Whistler, 2001; Pierre et al., 2009). The cAMP superactivation was known as the cellular basis of

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opioid withdrawal (Berger and Whistler, 2010). A phenomenon of downregulation of opioid receptors after chronic opioid treatment is a long-term-adaptive process that may result from degradation of internalized receptors and/or from a decrease of receptor synthesis. Changes in MOR number in response to chronic opioid treatment have long been speculated to directly contribute to receptor desensitization and the development of opioid tolerance (Connor et al., 2004).

Mitragyna speciosa (*M. speciosa)* Korth. (Rubiaceae) is native in southern regions of Thailand and northern region of Malaysia. In Thailand, this plant is known as “Kratom” and in Malaysia it is known as “Biak-biak” or “Ketum”. To date over 40 compounds have been isolated from the leaves of this plant (Adkins et al., 2011). In Malaysia and Thailand, *Mitragyna speciosa* leaves in the form of decoction has been traditionally used by the local people as a substitution therapy for chronic opioid treatment to manage the withdrawal symptoms especially on treatment substitution for morphine and heroin (Matsumoto et al., 2004; Vicknasingam et al., 2010). Mitragynine which is present in the aqueous extract or decoction is the most abundant of the 25 alkaloids found in this medicinal plant and it represent 66% (w/w) of the crude base (Takayama, 2004). Mitragynine is also known to be responsible for the substance's opioid effects (Yamamoto et al., 1999) and has shown to have the ability to reduce opioid withdrawal abstinence symptoms in animal models (Khor et al., 2011). In addition, *Mitragyna speciosa* is also utilized for common illnesses such as coughing, diarrhea, muscle pain, hypertension and to cure opioid addicts (Jansen and Prast, 1988). Although *Mitragyna speciosa* and mitragynine were claimed to have the potential to reduce the opioid tolerance and dependence, there is no report on cellular mechanisms for chronic opioid treatment.

In this study, the effects of cotreatment and pretreatment between morphine and mitragynine in forskolin-stimulation cAMP and MOR mRNA expression were investigated. We showed that mitragynine has lower opioid effect as compared to morphine. Mitragynine had also been able to reduce the up-regulation of cAMP level and downregulation of MOR mRNA expression in SK–N–SH cells after cotreatment with morphine for 24 h. Similar results were observed when cells were pretreated with morphine for 6 h and then exposed to mitraygnine for 6 h.

2. Materials and method

2.1. Materials

SK–N–SH neuroblastoma cells were purchased from American Type Culture Collection (ATCC, USA). Minimum Essential Medium, MEM, fetal bovine serum (FBS) and penicillin–streptomycin solution were obtained from Gibco (Invitrogen, USA). Dimethyl sulfoxides (DMSO), retinoic acid, forskolin and naloxone hydrochloride dehydrate were purchased from Sigma-Aldrich (USA). Morphine sulfate and methadone hydrochloride were purchased from Lipomed AG (Switzerland). Isobutylmethylxanthine (IBMX) and Calbiochem® cAMP Direct Immunoassay Kit, Colorimetric were purchased from Merck (Germany).

2.2. Plant material

The fresh leaves of *Mitragyna speciosa* Korth. (Rubiaceae) were collected from natural sources in Perlis, Malaysia. Authentication of plant material was carried out by botanist from Forest Research Institute Malaysia (FRIM) with a voucher specimen number PID 050412-01 and conserved at the Department of Bio-Screening, Malaysia Institute Pharmaceutical and Nutraceutical (IPharm) with code number IPHARM-49-35-C1.

2.3. Purification of mitragynine

The fresh leaves were dried using the drying oven at 50 °C for 4 days, 300 g powdered dried material was extracted with methanol for 72 h using soxhlet apparatus. Then filtered and evaporated under reduced pressure at 40–45 °C to obtain a methanol extract (90 g). An aliquot (90 g) of the methanol extract was dissolved in 10% (v/v) acetic acid, well shaken and left to stand for 24 h, then filtered to give the acidic filtrate, which was washed with n-hexane, made alkaline (pH 9) with 25% (v/v) ammonia solution and extracted with portions of chloroform. The chloroform extract was washed with dried over anhydrous sodium sulfate and evaporated to give crude alkaloid extract 3.5 g. The crude alkaloid was purified using SiO_2 column chromatography with eluent system n-hexane and ethyl acetate. The eluent was evaporated to dry and then was recrystallized with n-hexane and diethyl ether to give major compound mitragynine yields approximately 1.5 g (30%), which appeared as a single spot on a silica thin layer chromatography (TLC) analysis. Purified mitragynine was confirmed by HPLC and ^{1}H-NMR (400 MHz) analysis. The spectral data of mitragynine obtained from the present work are as follows: EI-MS *m*/*z* (relative intensity, %): 398 (M^+,41; $C_{23}H_3 \cdot N_2O_4$), 397 (28), 214 (16), 208 (17), 178 (100), 147 (97.2) and 132 (35.8). ^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.73 (1H,br–s,NH), 7.43 (1H, s, H-17), 6.99 (1H, t, $J=8.0$ Hz, H-11), 6.90 (1H, d, $J=8.0$ Hz, H-12), 6.45 (1H, d, $J=8.0$ Hz, H-10), 3.87 (3H, s, 9-OCH_3), 3.73 (3H, s, 17-OCH_3), 3.71 (3H, s, $COOCH_3$), 3.15 (1H,br–dd, $J=11.0$,2.0 Hz,H-3), 3.11 (1H, td, J=12.0, 2.7 Hz, 3.03 (1H, dt, $J=12.0$, 3.6 Hz, 2.93–2.99 (3H, m), 2.53 (2H, m), 2.45 (1H, dd, $J=11.2$, 2.8/2.9 Hz), 1.79 (2H, m), 1.62 (1H, m), 1.20 (1H, m) 0.87 (3H, t, $J=7.3$ Hz, 18-CH_3. ^{13}C-NMR (125 MHz, $CDCl_3$): δ 169.3 ($COOCH_3$), 160.6 (C-17), 154.5 (C-9), 137.2 (C-13), 133.7 (C-2), 121.8 (C-11), 117.7 (C-8), 111.5 (C-16), 107.9 (C-7), 104.2 (C-12), 99.7 (C-10), 61.6 (17-OCH_3), 61.3 (C-3), 57.8 (C-21), 55.3 (9-OCH_3), 53.8 (C-5), 51.4 ($COOCH_3$), 40.7 (C-20), 39.9 (C-15), 29.9 (C-14), 23.9 (C-6), 19.1 (C-19), 12.9 (C-18). Mitragynine obtained by this procedure was approximately 98% pure based on the average intensity ratio between the carbon signals of mitragynine and those of trace impurity observed in the baseline of the ^{13}C-NMR spectrum.

2.4. Cell culture and treatments

The human neuroblastoma cell line (SK–N–SH, ATCC # HTB-11; American Type Culture Collection, Manassas, VA) was used to measure the effect of cyclic AMP. SK–N–SH cells were maintained in Minimum Essential Medium, MEM, Gibco supplemented with 10% (v/v) fetal bovine serum, 100 U/ml penicillin and 100 mg/ml streptomycin (Invitrogen). SK–N–SH cells were grown as a monolayer in a humidfied incubator supplemented with 5% CO_2 at 37 °C and treated with retinoic acid (RA) at a concentration of 10 μM for 6 days to induce partial neuronal differentiation (Yu et al., 2003). Morphine and mitragynine dose response studies were conducted by removing the growth media and replacing with fresh growth media containing the following final concentrations of morphine; 0.1, 1, 10 and 100 μM or mitrtragynine; 0.1, 1, 10 and 50 μM. SK–N–SH cells were either vehicle treated or incubated with morphine for 24 h for each dose. As a negative control, cells were treated with an equivalent volume of vehicle in growth media. In order to determine if opioid receptors were mediating morphine's effects, mitragynine was cotreated with morphine for 24 h and pretreated with morphine 6 h then exposed with mitrgyanine 6 h incubation. The treatment groups were vehicle, morphine alone (50 μM), morphine (50 μM) with naloxone (50 μM), methadone (50 μM) or mitragynine (0.1, 1, 10 or 50 μM). All drugs were dissolved with 0.5% (v/v) DMOS. Finally, the supernatants were harvested for measuring the cAMP level. In a separate experiment, total RNA was isolated for Quantitative Reverse Transcription-PCR (qRT-PCR). All experiments were completed from at least three independent experiments for each time point.

2.5. Cell proliferation assay

Cell proliferation assay was carried out using the AlamarBlue Assay (Invitrogen, USA) following the manufacturer's protocol. Briefly SK–N–SH cells were plated into 96-well plates at a cell density of an approximately 3×10^4 cells/well and incubated 24 h at 37 °C. After 24 h incubation cells were treated mitragynine (3.93–1256.98 μM) and incubate for 72 h. Subsequently the medium was removed and replaced with fresh medium before adding 20 μl of resazurin into each wells. The cells were further incubated at 37 °C for another 4 h. The absorbance readings were determined at 540–570 nm is using En VisionTM 2104 Multilabel Reader (Perkin Elmer, USA).

2.6. Measurement of cyclic AMP accumulation

Treated and non-treated SK–N–SH cells in 24 well plates were assayed for the cAMP content after 24 h incubation move thusing the kit. Briefly, the medium was removed and cells were washed with PBS for three times to ree remaining drug. The medium was replaced with 0.4 ml of MEM medium containing 0.5 mM isobutylmethylxanthine. Isobutylmethylxanthine, a phosphodiesterase inhibitor is used to block the breakdown of cAMP in SK–N–SH cells, and were incubated for 30 min at 37 °C. Subsequently the culture medium was then removed and replaced with 0.4 ml of fresh medium containing 25 μM forskolin. Forskolin, an adenylyl cyclase activator commonly used to raise levels of cAMP in the cell physiology. After 10 min, the incubation medium was removed and cells were washed with PBS for several times. 0.5 ml of 0.1 N HCl was then added to lyse the cells and subsequently centrifuged at 2000*g* at 4 °C for 10 min, the supernatant was immediately assayed for cAMP using the enzyme immunoassay kit according to the manufacturer's instructions. Briefly, 100 μl of the preacetylated samples and 100 μl of cAMP standards were added into the wells of a mircoplate containing goat anti-rabbit IgG microplate. 50 μl of the blue cAMP conjugates and the yellow cAMP antibodies were sequentially added. The plate was incubated on a plate shaker at room temperature for 2 h at 500 rpm. Subsequently the contents of the wells were emptied and rinsed at least three times using 200 μl of wash solution. After the final wash, 200 μl of the substrate solution was added to every well and incubated at room temperature for 1 h. 50 μl of the stop solution was added to every well, and the optical density was read immediately at 405 nm with correction between 570 nm and 590 nm. The intracellular cAMP concentration was expressed as picomoles per milligram of protein and the graph was expressed as a percentage (%) of forskolin.

2.7. RNA isolation and cDNA synthesis

Total RNA was isolated using the RNeasy® Mini Kit (Qiagen, USA). Briefly, cells were homogenized by centrifugation using QIAshredder™ (Qiagen USA). The homogenized cells were transferred into RNeasy column and the total RNA was extracted following the manufacturer's protocol. The total RNA was quantified using Ultrospec 3100 pro Spectrophotometer (GE Healthcare, UK). The purity of RNA samples were electrophoresed on 1.5% (w/v) agarose total RNA quality and integrity. One microgram of total RNA was treated with DNAse-I and subsequently used for cDNA synthesis using Quantiscripts reverse transcriptase (RT). cDNA was then synthesized in a thermal cycler using the following cycling parameters: 2 min at 42 °C for genomic DNA elimination followed by 45 min at 42 °C and 3 min at 95 °C to inactivate the Quantiscript RT. All RT-reactions were stored at −20 °C.

2.8. Quantitative real-time PCR (qRT-PCR)

To quantify changes in human MOR messenger RNA (mRNA), the SYBR green I dye chemistry was employed. All primers were synthesized by Bioneer, Korea. qRT-PCR was performed using iScript™ One-Step RT-PCR Kit in the CFX96™ Real-Time System (Bio-Rad, USA) and the results were analyzed using CFX Manager™ Software, version 1.5 (Bio-Rad, USA). The annealing temperature of the primer sets (Table 1) for the human MOR (GenBank NM_000914) and housekeeping gene, human GAPDH (GenBank NM_002056.3) was optimized using gradient RT-PCR. This is followed by melting curve analysis to evaluate the specificity of each primer. PCR efficiency was carried out to evaluate the performance of the respective primers sets. In order to determine the qRT-PCR efficiency (rate at which a PCR amplicon is generated) and the dynamic range for both MOR and GAPDH genes, tenfold dilution series of cDNA (standard curve) starting from 100 ng to 1 ng, as a result efficiency for MOR and GAPDH were 98.9% and 95.4% respectively. The reaction mix was prepared in a total volume of 25 μl containing 12.5 μl 2X SYBR Green Reaction Mix, 0.75 μl (0.3 μM) of each primer, 0.5 μl of iScript Reverse Transcriptase, 9.5 μl of Nuclease-free H_2O and 1 μl of RNA template (100 ng). The optimized qRT-PCR amplification was carried out under the following conditions: cDNA synthesis at 50 °C for 10 min, RT inactivation at 95 °C for 5 min, and 38 cycles of denaturation at 95 °C for 10 s, annealing at 60 °C for 30 s and extension at 72 °C for 50 s. Relative quantification of MOR expression was calculated using the Pfaffl method (Pfaffl, 2001), where the expression level of MOR was normalized to GADPH as the reference gene. Three independent experiments were carried out and each experiment was performed in three analytical replicates.

2.9. Statistical analysis

The analysis of data was performed by means of one-way Anova for group comparison followed by Dunnet's test. Group differences were considered statistically significant at $*p < 0.05$.

Table 1
MOR and GAPDH primer sets used quantitative RT-PCR (qRT-PCR).

Target	Primer sequence (5′→3′)	Amplicon size	GenBank™ accession
MOR	Fwd: (590-609)n-CTTCAGCCATTGGTCTTCCT Rev: (689-668)-CAGTACCAGGTTGGATGAGAGA	106	NM_000914
GADPH	Fwd: (511-530)-CATGAGAAGTATGACAACAG Rev: (626-609)-ATGAGTCCTTCCACGATA	116	NM_002046

3. Result

3.1. *In vitro cell proliferation assay*

The cytotoxicity of mitragynine on SK–N–SH was determined using alamarBlue® assay. The dose response curve was plotted by plotting the percentage growth of cells vs. log concentration of the compound. As shown in Fig. 1A, the IC_{50} value of mitragynine was 77.268 μM. Thus, the mitragynine concentrations used for subsequent experiments were 0.1, 1, 10 and 50 μM.

3.2. *Morphine sulfate and mitragynine induced regulation of cyclic AMP in SK–N–SH cells*

SK–N–SH cells were treated with 0.1, 1, 10 and 100 μM morphine or 0.1, 1, 10 and 50 μM mitragynine for 5 min and 24 h. Treatment of cells with morphine for 5 min down-regulated the cAMP level. Result showed that concentrations at 10 and 100 μM of morphine gives the significant reduction in the camp level stimulated by forskolin as compared to cell only (control) (Fig. 2A). Meanwhile treatment of cell with mitragynine for 5 min showed 50 μM of mitragynine gives the significant reduction in the cAMP level stimulated by forskolin as compared to control (Fig. 2B). By contrast, cells treated with morphine for 24 h produced chronic morphine addiction as is shown by the increased in the cAMP level as compared with the cells stimulated by forskolin. These effects were observed when the cells were treated with morphine at higher dose levels, i.e. 10 and 100 μM (Fig. 2C). Interestingly, cells pretreated with mitragynine for 24 h also showed significant increase in cAMP level as compared to control in concentration between 10 μM and 50 μM (Fig. 2D). To determine the effect of incubation time on the effects of cAMP levels, a fixed dose of morphine (50 μM) was used to treat SK–N–SH cells in a time course manner. As shown in Fig. 2E, the percentage of the cAMP level content appeared to decrease from 30 s and reached a minimum level at 1 min before increasing again thereafter. However, the changes of cAMP content were not statistically significant until the incubation times reaches 6 h and above. Morphine at 50 μM produces a statistically significant increase in the percentage of cAMP content as compared to forskolin-treated cells at 6, 12 and 24 h of treatment. Therefore, the development of tolerance and dependence in SK–N–SH cells start from 6 h upward in 50 μM concentrations of morphine used in this experiment.

3.3. *Effect of co-treatment and pre-treatment of morphine sulfate and mitragynine on cAMP regulation*

In this study, the effect of mitragynine on a cAMP level and the interaction between mitragynine and morphine were investigated. SK–N–SH cells were treated with mitragynine and morphine together for 24 h. To determine if mitragynine will attenuate the increase in cAMP content produced by morphine, the SK–N–SH cells were cotreated with 50 μM of morphine and a series of concentration of mitragynine (0.1–50 μM of mitragynine). The agonist and antagonist opioids (methadone and naloxone respectively) were used as controls in this experiment. Methadone used in the treatment of opioid addiction has the capability to block the compensatory increase in intracellular cAMP in chronic opioid treatment and naloxone-precipitated cAMP overshoot observed after chronic treatment with morphine (Liu et al., 1999). As expected there was a clear reduction of forskolin-stimulated cAMP content in morphine and methadone-treated SK–N–SH cells. As for naloxone, there was a surge in cAMP content when the drug was co-treated with morphine. Mitragynine was shown to inhibit the forskolin-stimulated cAMP content induced by morphine at concentrations 0.1 and 1 μM (Fig. 3A). Similarly when SK–N–SH cells were pre-treated with morphine for 6 h, withdrawn and then re-applied with mitragynine for another 6 h. As shown in Fig. 3B, chronic morphine treatment (exposed and reapplied with morphine) and morphine withdrawal treatment increased the cAMP level as compared to forskolin stimulation only. Methadone and mitragynine (50 μM and 1.0 μM respectively) significantly reduced the cAMP level. It is also observed that mitragynine increased cAMP level in a dose dependent manner. The result showed that mitragynine has the potential to reduce the cAMP level of chronic morphine treatment at lower concentration.

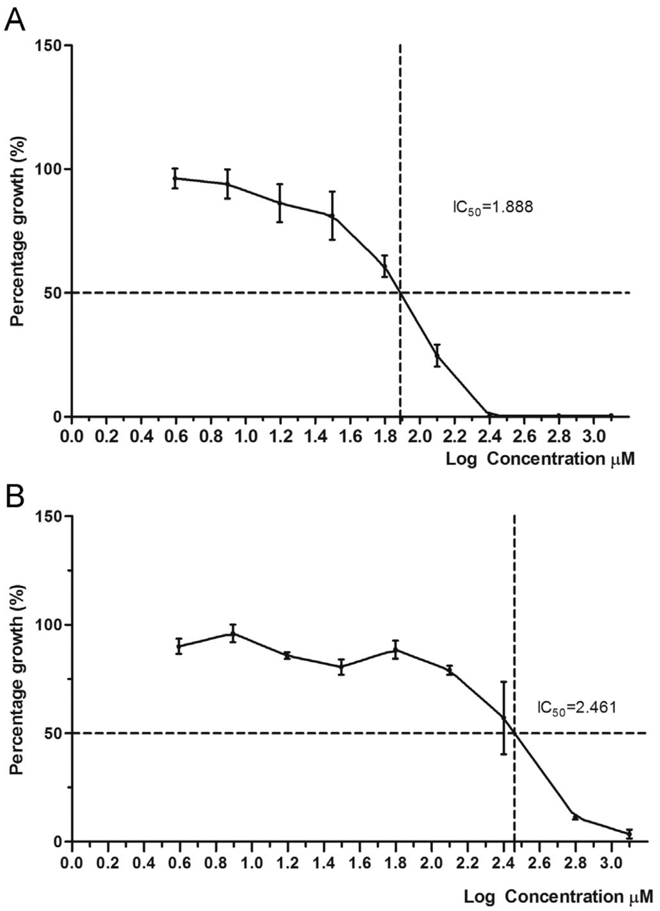

Fig. 1. The cytotoxicity effects of mitragynine and morphine on SK–N–SH cells. The cells were treated with a range of concentration 3.93–1256.98 μM and incubated for 72 h. The IC_{50} value of (A) mitragynine was 77.268 μM and (B) morphine was 212.9 μM. Data represent the mean ± S.D. of three independent experiments.

3.4. *Effects of retinoic acid (RA) on MOR mRNA expression*

The present study also investigated whether MOR mRNA expression is altered during differentiation. SK–N–SH cells were treated with a 10 μM RA for 6 days to induce differentiation cell. MOR mRNA levels were then examined and compared with undifferentiated cells cultured in vehicle 0.25% (v/v) DMSO (growth medium). The MOR mRNA level increased 1.65-fold (Fig. 4A) after RA-induced differentiation cells as compared to undifferentiated SK–N–SH cells.

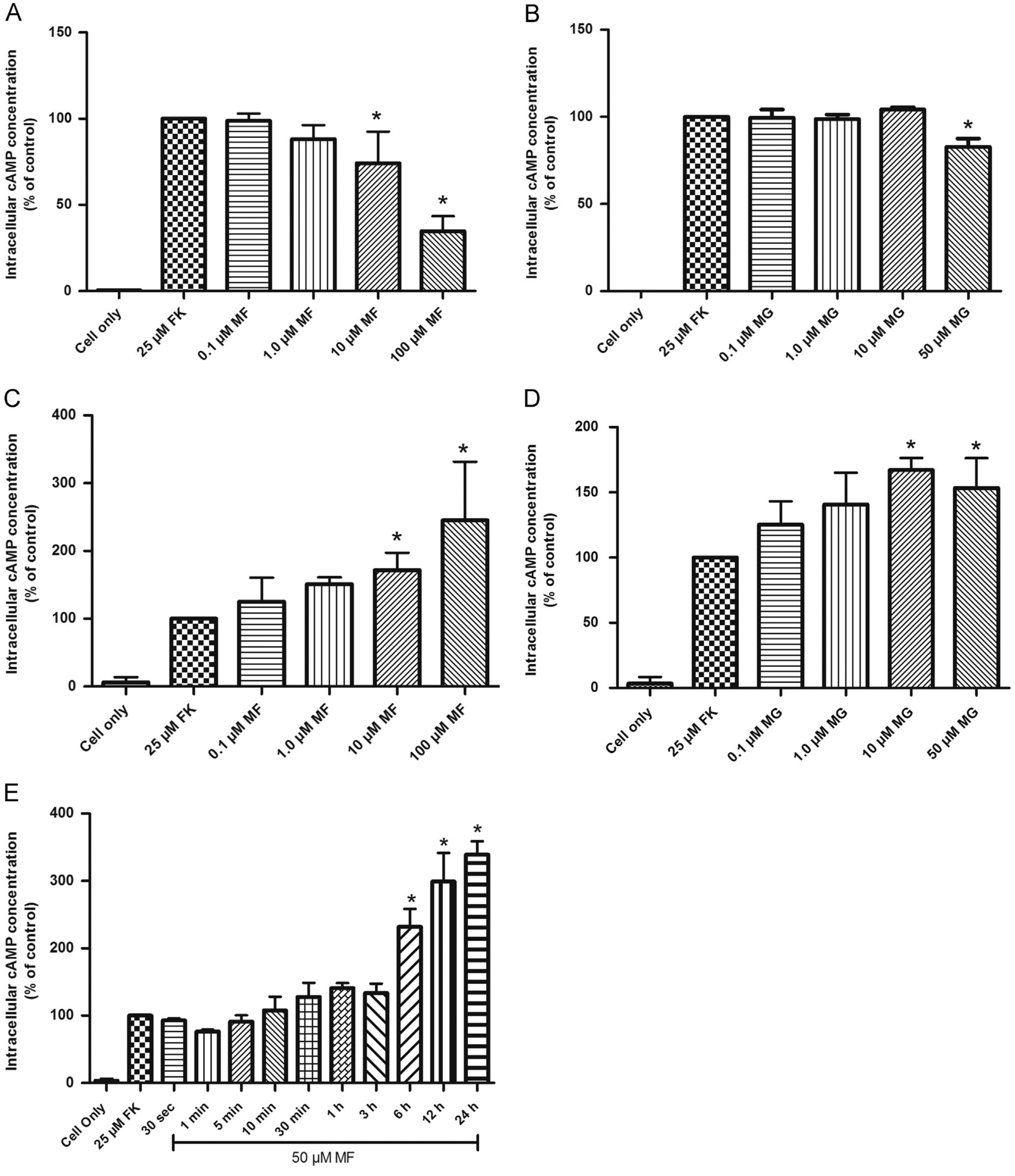

Fig. 2. Dose response effect of morphine (A) and mitragynine (B) on forskolin stimulation cAMP production after 5 min incubation. Dose response effect of morphine (C) and mitragynine (D) on forskolin stimulation cAMP production after 24 h incubation. Time response study of morphine on forskolin stimulation cAMP production (E). Three independent experiments were carried out and the data represent the mean ± S.D (MF = Morphine, MG = Mitragynine, and FK = Forskolin). Statistical analysis was carried out using a student Dunnet's test *$P \leq 0.05$ vs. 25 μM forskolin.

3.5. Morphine sulfate and mitragynine induced down-regulation of MOR in 24 h after treatment in SK–N–SH

The effect of 0.1, 1, 10 and 100 μM morphine or 0.1, 1, 10 and 50 μM mitragynine for 5 min and 24 h were also examined. Cells pretreated with morphine for 5 min showed no significant difference between morphine and differentiated cell (Fig. 4B). A similar effect was observed when the cells were pretreated with mitragynine for 5 min (Fig. 4C). Morphine at concentrations 1, 10 and 100 μM significantly down-regulated MOR 1.17-fold, 1.92-fold and 0.96-fold as compared to differentiated cells only at 24 h incubation (Fig. 4D). These results are consistent with the binding

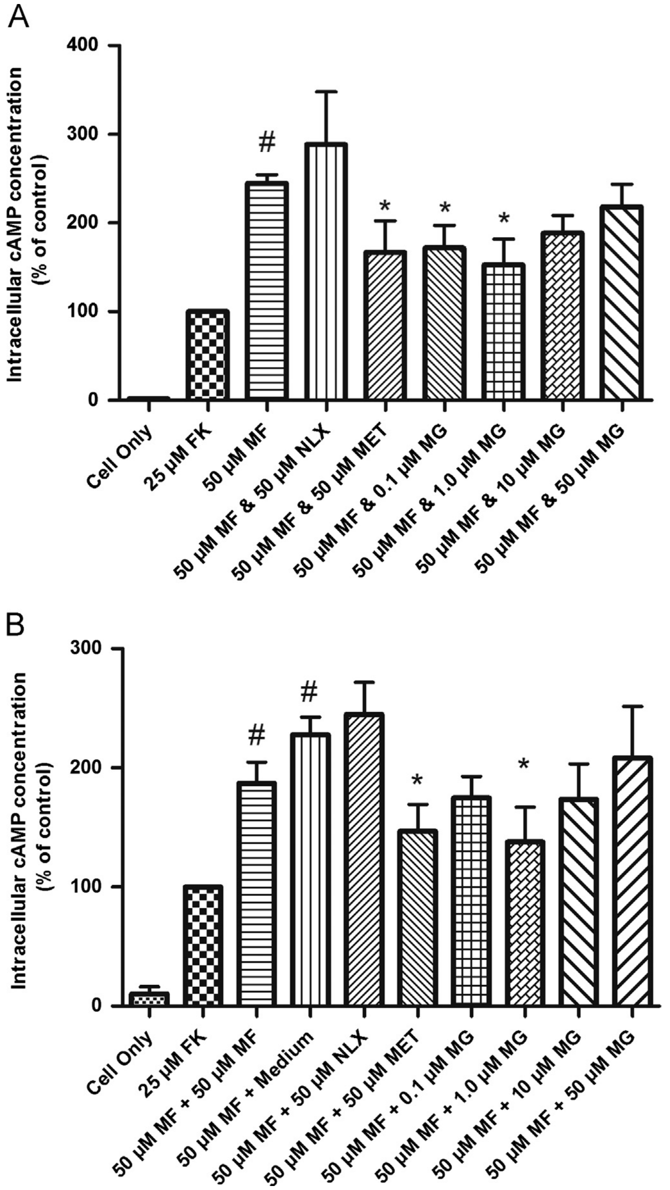

Fig. 3. Dose response effect of co-treatment (A) and pre-treatment (B) morphine sulfate and mitragynine on forskolin stimulation cAMP production after 24 h incubation. Three independent experiments were carried out and the data represent the mean ± S.D (MF=Morphine sulfate, NLX=Naloxone, MET=Methadone MG=Mitragynine, and FK=Forskolin) Statistical analysis was carried out using a student Dunnet's test #$P \leq 0.05$ vs. 25 μM forskolin and (A) *$P \leq 0.05$ vs. 50 μM MF, (B) *$P \leq 0.05$ vs. 50 μM MF +Media.

assay data previously reported by Yu et al. (2003). Interestingly, pre-treatment of cells with mitragynine showed significant down-regulation in the MOR mRNA expression 0.56-fold at a concentration of 50 μM (Fig. 4E).

3.6. *Effect of co-treatment and pre-treatment of morphine sulfate and mitragynine on MOR gene expression*

Further investigation was carried out to determine if cells treated with mitragynine would affect MOR mRNA expression in SK–N–SH cells. Interestingly cotreatment of mitragynine with morphine and methadone with morphine showed reduction in downregulation of MOR mRNA expression (Fig. 5A). Concentrations at 1 and 10 μM mitragynine were shown to significantly decrease the downregulation as compared to treatment with morphine only. Meanwhile treatment with naloxone totally block the downregulation of MOR mRNA expression (Fig. 5A) and this result is similar with previously reported by Yu et al. (2003). Cells pretreated with morphine for 6 h and exposed with mitragynine for another 6 h (Fig. 5B) showed no significant result but there is a indication that mitragynine has the potential to reduce the downregulation of MOR mRNA expression after chronic morphine treatment.

4. Discussion

Mitragyna speciosa is a tropical plant that is widely found in the rainforest of Malaysia and in the central and southern regions of Thailand and the traditionally used of this plant is to reduce and manage the drug withdrawal behavior (Vicknasingam et al., 2010). In the present study, the potential of mitragynine, a bioactive compound from *Mitragyna speciosa* as drug substitution therapy for chronic morphine treatment on a regulation cAMP level and expression MOR in neuroblastoma SK–N–SH was investigated.

It is well understood that, repeated exposure to drugs of abuse alters the amounts, and even the types, of genes expressed in specific brain regions (Nestler, 2004). In this aspect, altered expression of genes can mediate changes in the function of individual neurons and the larger neural circuits within which the neurons operate. Activation of any of the three opioid receptor subtypes produces common cellular actions. The most commonly reported actions include inhibition of adenylyl cyclase, activation of a potassium conductance, inhibition of calcium conductance, and an inhibition of transmitter release (Williams et al., 2001). Other observations studied in the actions of opioids are the activation of protein kinase C (PKC), the release of calcium from extracellular stores, the activation of the mitogen-activated protein kinase (MAPK) cascade, and the realization that receptor trafficking plays an important role in receptor function (Berger and Whistler, 2010).

In the present study, the changes in cyclic AMP and expression of MOR during the differentiation of SK–N–SH human neuroblastoma cells, the possible mechanisms involved in those changes and the differential effects of morphine and mitragynine were examined. Retinoic acid (RA) has been shown to induce differentiation of SK–N–SH and SHSY-5Y neuroblastoma cells although some phenotypic differences are noted in the differentiated cells (Sidell et al., 1986; Preis et al., 1988; Jenab and Inturrisi, 2002; Liang et al., 2009). Here, RA was used to induce differentiation of SK–N–SH cells for 6 days treatment. A previous report showed that differentiation of cells by RA can greatly enhance the ability of prostaglandin E_1 to stimulate adenylyl cyclase (AC) and the inhibition of stimulated AC by opioids. The opioid effect was attributed largely to the μ-receptor rather than the δ-receptor (Yu et al., 1990). Therefore, the RA treated human neuroblastoma cells are useful to study the efficacy and tolerance of narcotic analgesics. In the present study, treatment with a 10 μM RA to differentiated cells increased the MOR mRNA expression as compared to undifferentiated cells.

The short and the long term effects of morphine on forskolin-stimulated cAMP production using different concentrations of morphine were examined. It was found that the short-term exposure (5 min) with morphine inhibited the adenylyl cyclase activity and reduces cAMP production, whereas long-term exposure (24 h) has contributed to the superactivation of adenylyl cyclase, manifested by a rebound of cAMP production. It has been reported that chronic morphine exposure can affect the regulation of MOR in neuroblastoma cell (Yu et al., 2003; Mohan et al., 2010). The time dependent study showed that the inhibition of cAMP started from 30 s until 10 min and the increase in cAMP level starts from 6 h until 24 h in 50 μM concentration of morphine sulfate. This suggests that development of chronic morphine

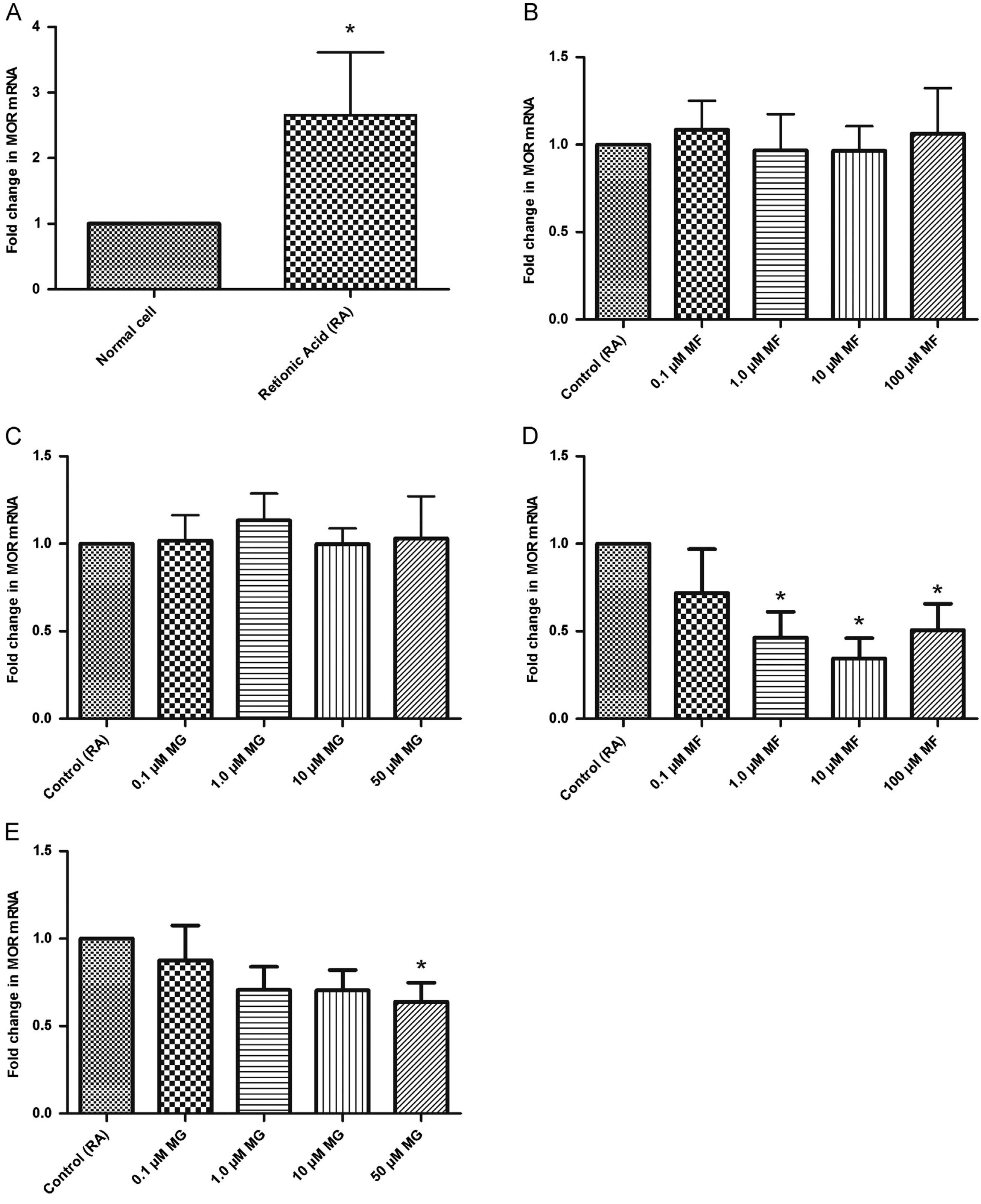

Fig. 4. (A) Effect of retinoic acid after 6 days treatment on mRNA expression MOR. Dose response effect of morphine (B) and mitragynine (C) on mRNA expression MOR after 5 min incubation. Dose response effects of morphine (D) and mitragynine (E) on mRNA expression MOR after 24 h incubation. Three independent experiments were carried out and the data represent the mean ± S.D (MF=Morphine sulfate). Statistical analysis was carried out using a student Dunnet's test *$P \leq 0.05$ s. Control (RA).

tolerance and dependence starts from 6 h onward. In another observation, treatment with morphine showed down-regulation of the MOR mRNA expression in SK–N–SH cells after 24 h treatment, confirming previous studies showing decreased radioligand binding of opioid ligands (Horner and Zadina, 2004) or MOR mRNA down-regulation (Yu et al., 2003) in the same or other neuroblastoma cell lines after chronic morphine exposure. An up-regulation of the cAMP pathway and down-regulation of MOR mRNA expression are known to the important adaptive changes induced by chronic exposure to opiate and is related to

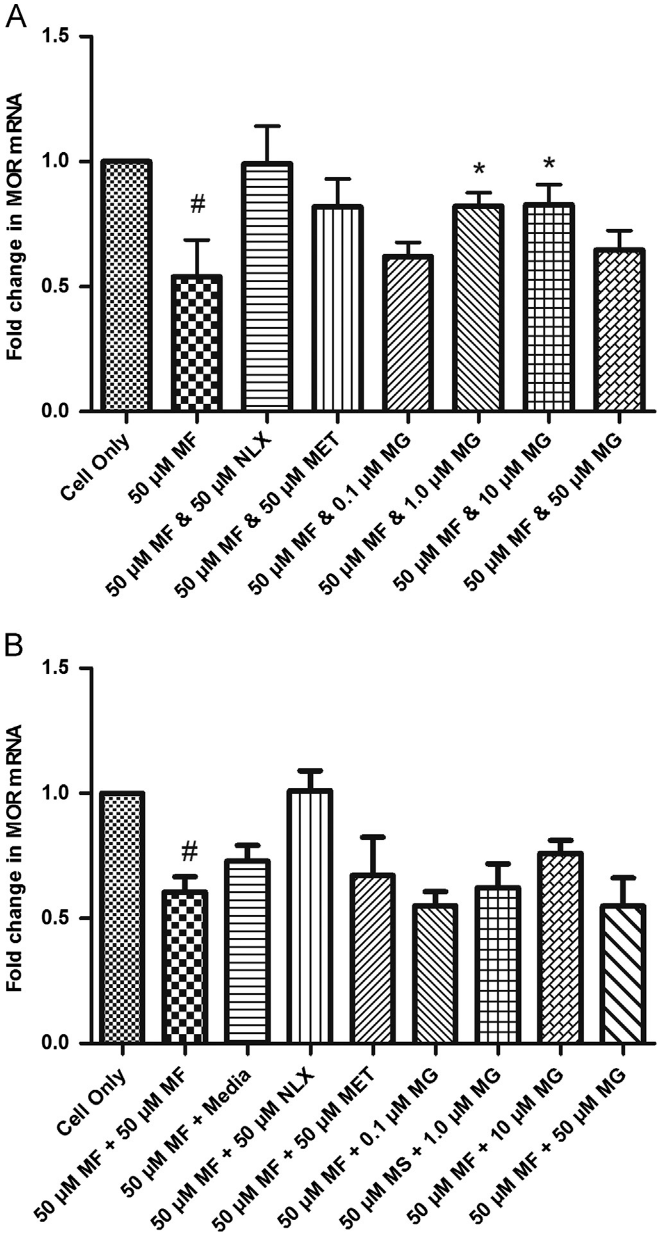

Fig. 5. Dose response effect of co-treatment (A) and pre-treatment (B) morphine sulfate and mitragynine on mRNA expression MOR. Three independent experiments were carried out and the data represent the mean ± S.D (MF=Morphine, NLX=Naloxone, MET=Methadone and MG=Mitragynine) Statistical analysis was carried out using a student Dunnet's test #$P \leq 0.05$ vs. Control (RA) and (A) *$P \leq 0.05$ vs. 50 μM MF, (B) *$P \leq 0.05$ vs. 50 μM MF+Media.

tolerance and dependence, which have been demonstrated at the level of individual neurons chronic exposure of SK–N–SH and SH-SY5Y cells, which express abundant MOR (Yu et al., 2003; Mohan et al., 2010). Mitragynine was reported to have potential as a substitution therapy for chronic morphine treatment (Babu et al., 2008). We also studied the effect of mitragynine alone on cAMP production and MOR mRNA expression after 24 h treatment. Interestingly, the application of mitragynine also showed similar effects in increasing the cAMP level and MOR down-regulation but the effect was lower as compared to morphine. The results of this study indicated that mitragynine has a lower opioid effect.

The effect of cAMP production and MOR mRNA expression in SK–N–SH cells after cotreated and pretreated of morphine with mitragynine were measured. The result of the present studies showed that cotreatment of mitragynine with morphine reduced the level of cAMP as compared to cell treated with morphine alone. A similar effect was observed when cells were pretreated with morphine for 6 h then exposed with mitragynine for another 6 h. Methadone was used as a positive control in this study. The result showed that methadone significantly reduced the cAMP level on forskolin stimulation in SK–N–SH cell. Furthermore, incubation with methadone during chronic exposure to morphine substantially prevented morphine-induced spontaneous overshoot. Methadone cotreated with morphine showed reduction in cAMP level production stimulated by forskolin in 78% as compared with morphine alone. Similarly pretreated with morphine for 6 h then exposed with methadone for another 6 h, methadone significantly reduced the cAMP level in 81% as compared to withdrawal morphine. These results suggest that methadone can prevent chronic morphine treatment-induced adaptive sensitization or overshoot of adenylate cyclase activity (Liu et al., 1999). Methadone and morphine are similar opioid receptor agonists but methadone has a lower dependence potential than morphine and is effectively used in the treatment of opioid addiction, whereas morphine induces dependence (Berger and Whistler, 2010). Treatment with naloxone was showing an increasing camp level as compared with morphine confirming in previously reported that addition of naloxone led to a further increase in cAMP accumulation naloxone-precipitated cAMP overshoot (Liu et al., 1999). Meanwhile 0.1 and 1 μM mitragynine cotreated with morphine significantly reduced the cAMP level as compared to morphine only in 73% and 92% reduction respectively. A similar effect was observed when cells were pretreated with morphine for 6 h then exposed to mitragynine another 6 h. The result showed that after morphine withdrawal for 6 h, 10 μM mitragynine significantly reduced the cAMP level as compared morphine withdrawal.

On the other hand, MOR mRNA expression showed that cotreatment and pretreatment of cells with morphine and mitragynine seemed to reduce the downregulation of mRNA as compared to treatment with morphine only. Cotreatment with morphine and mitragynine significantly reduced the downregulation of MOR mRNA expression at the concentrations of mitragynine at 1 μM and 10 μM. Meanwhile there was no significant difference in the pretreatment study. Previous report has shown mitragynine bound to three opioid receptors which are μ, δ and κ receptor (Taufik Hidayat et al., 2010). Mitragynine also has been shown to have high selectivity and affinity to opioid receptor especially MOR (Tsuchiya et al., 2002). Based on these different binding mechanisms, mitragynine has the potential to be used as an agent for opioid addiction treatment. The present study has the limitation to understand the mechanistic effect of activation of the cAMP level after chronic morphine treatment. Therefore, it will be interesting to study further the effect of mitragynine at the desensitization of opioid receptor and cAMP-response element binding protein (CREB).

In summary, these findings suggest that mitragynine could potentially attenuate the tolerance and dependence of chronic morphine treatment by reducing the up-regulation of cAMP level as well as reducing the down-regulation of MOR at a lower concentration of mitragynine. The findings also provide an understanding of the effect of mitragynine on the cellular basis of substitution therapy for opioid addiction. It also provides insight into the mechanisms of dependence and tolerance, thereby facilitating further studies on the design and development of highly effective and addictive substitute drugs for the therapy of opioid addiction.

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